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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Rabbani et al.
Serial No.:	08/978,636
Filed:	November 25, 1997
For:	**NON-NATIVE POLYMERASE ENCODING NUCLEIC ACID CONSTRUCT**

Group Art Unit: 1635

Examiner: J. Schultz

527 Madison Avenue(9th Floor)
New York, New York 10022
February 15, 2005

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT UNDER 37 C.F.R. §1.111
(IN RESPONSE TO THE JULY 15, 2004 OFFICE ACTION AND DECEMBER 9, 2004 ADVISORY ACTION)

Dear Sirs:

This is a response to the Office Action issued on July 15, 2004 and the Advisory Action issued on December 9, 2004 in connection with the above-identified application.

AMENDMENTS TO THE SPECIFICATION

Please amend the "BRIEF DESCRIPTION OF THE DRAWINGS" as follows:

--BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the localized attachment of ligands and other moieties to a nucleic acid construct by incorporation into a nucleic acid primer.

FIGURE 2 depicts the dispersed attachment of ligands to a nucleic acid construct by extension from a modified nucleic acid primer.

FIGURE 3 illustrates the dispersed attachment of ligands to a nucleic acid construct by synthesis of a complementary RNA strand that utilizes modified ribonucleotide precursors.

FIGURE 4 illustrates the localized attachment with a nucleic acid construct by hybridization of a gapped circle with a modified nucleic acid moiety that also contains useful moieties incorporated into a 3' tail.

FIGURE 5 illustrates the preparation of a gapped circle such as shown in FIGURE 4.

FIGURE 6 illustrates the localized attachment with a nucleic acid construct by hybridization of a gapped circle with a modified nucleic acid moiety with an unmodified 3' tail to which has been hybridized a nucleic acid with useful ligands incorporated thereinto.

FIGURES 7 AND 8 show the process for introducing a segment of RNA into a cell by means of a modified primer whereby the RNA will be transformed in vivo into a double-stranded DNA segment.

FIGURES 9 AND 10 show the process for introducing a segment of RNA into a cell by means of modified primers whereby the RNA will be transformed in vivo into double-stranded DNA segments.

FIGURE 11 illustrates a process for introducing a segment of single stranded DNA having modified nucleotides as part of its sequence.

FIGURE 12 illustrates the fate of the modified single-stranded DNA from Figure 11 after it has been introduced into a cell.

FIGURE 13 illustrates a process for introducing a segment of double stranded DNA having modified nucleotides as part of the sequence on each strand.

FIGURE 14 illustrates a divalent antibody binder with one portion having an affinity for binding a retroviral particle, and the other portion having an affinity for binding the CD34 antigen.

FIGURE 15 shows the covalent attachment of DNA to each portion of an F(ab')2 antibody fragment with an affinity for the CD34 antigen.

FIGURE 16(A) depicts the covalent attachment of DNA to an adenovirus binding portion of a divalent antibody in order to promote the binding of an AAV vector DNA molecule to a CD34 receptor.

FIGURE 16(B) is the same depiction as in FIGURE 16(A) except that F(ab') fragments are used instead of complete antibody proteins.

FIGURE 17 illustrates a monovalent antibody to an adenovirus spike protein with one portion being modified by covalent attachment of DNA that can bind an adenovirus associated virus (AAV) vector DNA molecule through hybridization and the other portion being modified by the covalent attachment of an oligolysine modified by the attachment of lactyl groups.

FIGURE 18 shows a monovalent antibody to an adenovirus spike protein in which each portion of the antibody has been modified by the covalent attachment of lactosylated DNA molecules which are bound to an AAV vector DNA by means of hybridization.

FIGURES 19 AND 20 describe the synthetic steps for producing a reagent that is useful for attaching nucleic acid moieties to an antibody.

FIGURE 21 depicts a process for mutlimerization of F(ab')2 antibody fragments by hybridization of nucleic acid homopolymers (polynucleotide sequences shown in SEQ ID NOS 52-54, from left to right).

FIGURE 22 depicts a process for multimerization of insulin molecules by hybridization of nucleic acid homopolymers (polynucleotide sequence shown in SEQ ID NO 52).

FIGURE 23 depicts a process for multimerization of insulin molecules by hybridization of nucleic acid heteropolymers with a binding matrix.

FIGURE 24 shows the introduction of an SV40 intron sequence that reconstitutes appropriate signals for in vivo splicing and production of a normal mRNA transcript for T7 RNA polymerase (polynucleotide sequences shown in SEQ ID NOS 2, 55, 3, 4, 56, 57, 5, 6, 58, 59, 7, 8 and 9, respectively, in order of appearance).

FIGURE 25 shows the process of the intron introduction and subsequent construction of a T7 expression vector.

FIGURE 26 shows the oligomers (SEQ ID NOS 10-17, respectively, in order of appearance) and their products used for the synthesis of the SV40 intron containing T7 RNA polymerase coding sequence.

FIGURE 27 depicts the process for the introduction of nucleotide sequences (SEQ ID NOS 10-11, 18-27, respectively, in order of appearance) for the nuclear localization signal.

FIGURE 28 is a comparison of the 5' ends of the nucleotide sequence for the normal T7 RNA polymerase (SEQ ID NOS 28-29) and a T7 RNA polymerase with sequences inserted for a nuclear localization signal (SEQ ID NOS 30-31).

FIGURE 29 shows the process for the assembly of PCR generated fragments by cloning methods to assemble a clone that directs the synthesis of an intron containing T7 RNA polymerase transcript.

FIGURE 30 shows the sequences for HIV antisense sequences (SEQ ID NOS 32-39, respectively, in order of appearance) and the process for their cloning into T7 directed transcription units.

FIGURE 31 shows the cloning steps for the combination of T7 directed antisense into a clone that contains the intron containing T7 RNA polymerase.

FIGURE 32 shows the DNA sequences (SEQ ID NOS 40-41, respectively, in order of appearance) and subsequent cloning steps for making a protein expression vector.

FIGURE 33 shows a process for a combination of the polylinker sequence (SEQ ID NOS 42-43, respectively, in order of appearance) from FIGURE 32 and a T7 promoter and a T7 terminator for making a T7 directed protein expression vector.

FIGURES 34 AND 35 depicts the design of a primary nucleic acid construct that will function as a production center to generate single stranded antisense DNA.

FIGURE 36 depicts the design of a primary nucleic acid construct that will generate a secondary nucleic acid construct capable of directing transcription.

FIGURES 37 AND 38 depict the design of a primary nucleic acid construct that will generate a double hairpin production center (secondary nucleic acid construct).

FIGURE 39 depicts the design of a primary nucleic acid construct that will generate a production center (secondary nucleic acid construct) capable of inducible suicide.

FIGURE 40 depicts the design of a primary nucleic acid construct that will use tRNA primers in vivo to make secondary nucleic acid constructs capable of transcription.

FIGURE 41 depicts the process of excision of normal sequences from a U1 transcript region and replacement with novel sequences.

FIGURE 42 shows the oligomer sequences (SEQ ID NOS 44-51, respectively, in order of appearance) for making HIV antisense sequences and the insertion of these oligomers as replacement for a portion of the U1 transcript sequence in a clone containing a U1 operon.

FIGURE 43 is a computer generated secondary structure prediction for U1 transcripts with HIV antisense sequence substitutions (SEQ ID NOS 60-63, respectively, in order of appearance).

FIGURE 44 depicts the cloning process for making of a clone that contains multiple HIV antisense containing U1 operons.

FIGURE 45 depicts the cloning steps for constructing a clone that contains multiple independent HIV antisense containing T7 directed transcripts.

FIGURE 46 shows the final structures of the multiple operon constructs described in FIGURES 44 and 45.

FIGURE 47 depicts the cloning steps for insertion of multiple T7 antisense operons into a vector coding for the T7 intron containing RNA polymerase.

FIGURE 48 represents flow cytometry data measuring binding of anti-CD4+ antibody to HIV resistant U937 cells.

FIGURE 49 shows PCR amplification of the gag region indicating the absence of HIV in viral resistant cell line (2.10.16) after challenge.

FIGURE 50 depicts a model system for testing the potential inhibition of HIV antisense sequences by using beta-galactosidase activity as an indicator.

FIGURE 51 is a table of data demonstrating the effect of the HIV antisense sequence upon beta-galactosidase activity by enzyme assays as well as in situ assays.

Please amend page 124, lines 12-16 as follows:

(ii) Synthesis of peptides for addition into the DNA primer

The sequence coding for the Fusogenic Peptide (Gly-Phe-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Leu-Glu-Gly-Gly-Trp-Glu-Gly-Met-Ile-Ala-Gly) (SEQ ID NO:1) and the sequence coding for the Nuclear Localisation Peptide are synthesized chemically with an additional cysteine group added onto the carboxy terminus of each.

Please amend page 144, line 1 to page 145, line 3 as follows:

Annealing is done 0.2M NaCl, 0.05M Tris HCl (pH 7.8), 1 mM EDTA. FIG. 21 shows the overall outline of the process. In the last step shown in FIG. 21, (a) shows an example where both the A homopolymer and the T homopolymer are short enough that there is essentially only one of each type of molecule binding together in a 1:1 ratio (SEQ ID NOS 52-54). The (b) diagram shows the situation where the A homopolymer was synthesized such that its much longer than the T homopolymer; in this situation, larger numbers of antibodies can be linked together into complexes.

Example 17

Preparation of a Multimeric Insulin by Means of Nucleic Acid Hybridization

Oligo T with a primary amino group (prepared as described earlier) is reacted in 0.7M LiCl , 0.1M sodium bicarbonate buffer, pH 7.8 and 30% dimethyl formamide with a 3-fold excess of suberic acid bis (N-hydroxysuccinimide) ester for 15 minutes at room temperature. The pH was then lowered to 5.0 by the addition of 2M acetic acid and the excess of active ester was extracted twice with n-butanol. The nucleic acid was precipitated with 4 volumes ethanol at -70°C. and the pellet after centrifugation was dissolved in cold 0.7M LiCl in 0.1M sodium bicarbonate solution (pH 7.8), solid insulin was added in 1:1.2 ratio and the conjugation was allowed to take place at 4°C. overnight. The product is separated from the reactants by molecular sieving chromatography on G75 columns. A multimeric complex is formed by the hybridization of the T-tailed insulin molecules (SEQ ID NO 52) with a Poly A binder as described earlier. The steps in this Example are shown in FIG. 22.

Please amend page 148, line 10 to the bottom of page 153 as follows:

(C) Synthesis of the individual segments used for the fusion.

The T7 RNA polymerase is encoded by bases 3171-5822 in the T7 genome (Dunn and Studier, 1983 J. Mol. Biol. 166: 477) and this sequence is available in Genbank as Accession #'s V01146, J02518 or X00411. Based upon this information, six different oligos were synthesized. The use of these oligos and their sequences are given in FIG. 26. TSP 1 (SEQ ID NO 10) and TSP 2 (SEQ ID NO 11) were annealed together by a 12 bp complimentary sequence and extended to form a completely double-stranded DNA molecule (FIG. 27). Conditions were as follows: 150 pM of TSP 1 (SEQ ID NO 10), 150 pM of TSP2 (SEQ ID NO 11), 1* NEB Buffer #2 (New England Biolabs, Inc.), 200 uM dNTP and 13 units of Sequenase v2.0 (U.S. Biochemicals, Inc) for 75 minutes at 37°C. TSP 3 (SEQ ID NO 12) and 4 (SEQ ID NO 13) were used in a PCR reaction (Saiki et al. 1985 Science 230, 1350) with T7 genomic DNA as a template to synthesize the "Left" fragment. Reagent conditions were as follows: 100 ul volume containing 100 ng T7 template (Sigma Chemical Co.), 1 uM TSP 3 (SEQ ID NO 12), 1 uM TSP 4 (SEQ ID NO 13), 1 mM MgCl2, 1* PCR buffer, 250 uM dNTP, 2.5 units of Taq DNA Polymerase. Temperature cycling conditions were: 16 cycles of (1) 50 seconds at 94°C. (2) 25 seconds at 50°C. and (3) 3 minutes at 72°C. The same conditions were used to form the "Right" end fragment with Oligomers TSP-5 (SEQ ID NO 14) and TSP-6 (SEQ ID NO 15) except that due to the length (over 2 kb) of the expected product, 2.5 units of Taq Extender (Stratagene, Inc) was added and the Taq Extender buffer substituted for the normal PCR buffer. INT-1 (SEQ ID NO 16) and INT-2 (SEQ ID NO 17) were used together in a PCR reaction to form the Intron piece. Conditions were the same as those used for synthesizing the "Left" fragment of T7, except that a clone of SV40 was used as the template and due to the smaller size of the amplicon, the cycle conditions were only 1' at 72°C. for the extension time. FIG. 27 shows the synthesis of the short double stranded piece of DNA made by extension of oligos TSP 1 (SEQ ID NO 10) and TSP

2 (SEQ ID NO 11) and its combination with the left end of the TSP 3/TSP 4 PCR product to generate the complete (NLS+) T7 RNA polymerase (SEQ ID NOS 30-31). The resultant nucleic and amino acid sequences are given in FIG. 28 for the construct given in this example as well as the normal wild type T7 RNA polymerase sequences (SEQ ID NOS 28-29).

Thus, the modifications carried out at the 5' end during this construction process were:

a) The sequence around the ATG start codon was changed to give a Kozak consensus sequence (Kozak 1984 Cell 44: 283) to increase efficiency of translation of the gene product. This change had previously been introduced into the T7 RNA polymerase coding sequence.

b) The fusion of the TSP1 /TSP2 (SEQ ID NOS 18-19) extension product to the TSP3/TSP4 PCR introduces a 9 amino acid insertion between bases 10 and 11 in the normal T7 RNA polymerase protein sequence. This sequence has previously been shown to be a signal for transportation to the nuclease by Kalderone et al. (1984 Cell 39: 499) and had been introduced into T7 RNA polymerase by Lieber et al., (1989) as a substitute for the first 10 amino acids and inserted into an artificially created EcoR1 site by Dunn et al., (1988). The method used in this Example to introduce the Nuclear Localisation Signal (NLS) was designed to minimize perturbations to the normal structure of the protein. The codons for the amino acids coding for the NLS are indicated as larger type size in FIG. 28

(D) Combination of pieces to form the final construct of the T7 RNA polymerase gene in a eucaryotic expression vector

FIG. 29 shows the various steps used for this process. For ease of use, each of the three pieces (PCR #1, PCR #2 and PCR #3) was cloned into a plasmid vector (PCR II) using the TA cloning kit and following the manufacturer's instructions (Invitrogen, Inc.).

PCR #1 (the left end of the T7 RNA polymerase) was cloned into PCR II to create pL-1 (SEQ ID NO 40). This construct was then digested with BsmB1 and Spe I to excise out the PCR product and the TSP1 /TSP2 Extension product (SEQ ID NOS 18-19, shown in detail in FIG. 27) was digested with Eco R1 and Bsa I. Due to the design of the primers, the single-stranded tails created by BsmB1 and Bsa I are complimentary to each other and ligation of these pieces forms a single piece with an EcoR1 tail at one end and a Spe I tail at the other end. Digestion of the M13 vector, mp18, with EcoR1 and Xba I allows insertion of the EcoR1/Spe I piece to form pL-2 (SEQ ID NO 41).

PCR #2 (the SV40 Intron) was cloned into PCR II to form pINT-1. This construct was digested with EcoR1 and Spe I and transferred into the M13 vector (mp18 digested with EcoR1 and Xba I) to form pINT-2.

PCR #3 (the right end of the T7 RNA polymerase) was cloned into PCR II to create pR-1. This construct was digested with Eco R1 and Spe I and then self-ligated to form pR-2. This step was added to eliminate extra EcoR1 and Spe I sites present in pR-1.

As described in FIG. 25, the elements in pL-2 (SEQ ID NO 41), pINT-2 and pR-2 are fused together to form the complete intron-containing T7 RNA polymerase. This was accomplished by digestion of pL-2 (SEQ ID NO 41) with BsmB1 and Bsa I; pINT-2 with BsmB1; and pR-2 with Bsal and Spe I. Ligation of these three inserts together forms a single fragment that has one end compatible with a Hind III end and the other

end compatible with Spe I. This fragment was cloned in the same step into pRc/RSV (from Invitrogen, Inc.) that had been previously digested with Hind III and Spe I. As shown in FIG. 29, this final product is pINT-3. This particular eucaryotic vector was chosen since it had been shown previously that the RSV promoter is especially active in hematopoietic cell lines. Also, the ligation of the Hind III end from pRcRSV to the end created from the BsmB1 digestion of pL-2 (SEQ ID NO 41), does not reconstitute the Hind III site in pINT-3, the final product.

E) Antisense sequences

Three different targets in the HIV genome were chosen as test targets for Antisense: (A) the 5' common leader, (B) the coding sequence for Tat/Rev and (C) the splice acceptor site for Tat/Rev. Antisense to (A) was derived from a paper by Joshi et al. (1991 J. Virol. 65,5534); Antisense to (B) was taken from Szakiel et al. (1990 Biochem Biophys Res Comm 169, 213) and the Antisense to (C) was designed by us. The sequences of the oligos and their locations in the HIV genome are given in FIG. 30. Each oligo was designed such that annealing of a pair of oligos gives a double-stranded molecule with "sticky ends" that are compatible with a Bam H1 site. The oligos were also designed such that after insertion into a Bam H1 site, only one end of the molecule would regenerate the Bam H1 site, thus orientation of the molecule could easily be ascertained. The resultant clones were termed pTS-A, pTS-B and pTS-C for the anti-HIV sequences A, B and C respectively.

F) Cloning of T7 terminator

The sequence for termination of transcription by the T7 RNA polymerase is encoded by a sequence between the end of the gene 10b protein at base number 24,159 and the start codon of the gene 11 product at base number 24,227 in the T7 genome (Dunn and Studier 1983 J. Mol. Biol. 166, 477 Genbank Accession #'s V01146, J02518 or X00411. Based upon this information, TER-1 (SEQ ID NO 38) and TER-2 (SEQ ID NO 39) were synthesized (Sequences given in FIG. 30) and used in a PCR

amplification reaction to obtain a double-stranded 138 bp piece that contained the T7 sequences from 24,108 to 24,228 with an Xba I site added at one end and a Pst 1 site added to the other. The reagent conditions for amplification were as described for the TSP3/TSP4 reaction but the temperature cycling conditions were: 16 cycles of (1) 50 seconds at 94°C. (2) 25 seconds at 50°C. and (3) 1 minute at 72°C. As shown in FIG. 30, the terminator piece was cloned into the PCR II vector and then after Xbal/Pst I digestion it was transferred into an M13 vector.

Please amend page 162, line 22 to page 163, line 7 as follows:

After digestion with Bcl 1 and Bsp E1, a 49 base pair segment is eliminated from the U1 transcript portion of the gene. The oligo pairs have been designed to form sticky ends compatible with the Bcl/Bsp ends in the plasmid. Ligation of each of the pairs of Oligos (HVA-1 (SEQ ID NO 44) +HVA-2 (SEQ ID NO 45), HVB-1 (SEQ ID NO 46) +HVB-2 (SEQ ID NO 47) and HVC-1 (SEQ ID NO 48) +HVC-2) (SEQ ID NO 49) created pDU1-A with an insertion of 72 bp, pDU1-B with an insertion of 66 bp and pDU1-C with an insertion of 65 bp. As a control, two oligomers (HVD-1 (SEQ ID NO 50) and HVD-2 (SEQ ID NO 51)) with sequences unrelated to HIV were also inserted into the U1 operon to create pDU1 which contains an insertion of 61 bp.

Please amend page 163, line 16 to page 164, line 2 as follows:
As described earlier, the design of the cloning method should allow the insertion of novel sequences that would still allow the utilisation of signals provided by the U1 transcript for nuclear localisation of Anti-sense sequences. To test whether the insertion of the sequences described above resulted in unintended changes in the U1 region responsible for re-importation of the U1 transcripts a computer analysis was done to compare the predicted structures for the normal U1 and the chimeric novel molecules using the MacDNASIS program (Hitachi, Inc.). In FIG. 43 it can be seen

that despite changes in the 5' end (where the new sequences have been introduced) loops III and IV as well as the Sm region remain undisturbed (SEQ ID NOS 60-63).

CLAIM AMENDMENTS

Claims 1-244 (cancelled)

245. (currently amended) A nucleic acid construct which comprises a nucleic acid sequence which encodes a non-eukaryotic polymerase, said sequence encoding said non-eukaryotic polymerase further comprises an intron, ~~and contains a non-native intron~~, wherein said polymerase is expressed solely in a eukaryotic cell and said polymerase is capable of producing more than one copy of a nucleic acid sequence from said construct when introduced into a eukaryotic cell.

246. (previously presented) The construct of claim 245, further comprising a recognition site for said polymerase.

247. (previously presented) The construct of claim 246, wherein said recognition site is complementary to a primer for said polymerase.

248. (previously presented) The construct of claim 247, wherein said primer comprises transfer RNA (tRNA).

249. (previously presented) The construct of claim 245, wherein said non-eukaryotic polymerase is selected from the group consisting of RNA polymerase, DNA polymerase, reverse transcriptase, and a combination thereof.

250. (previously presented) The construct of claim 249, wherein said RNA polymerase is a bacteriophage RNA polymerase.

251. (previously presented) The construct of claim 250, wherein said bacteriophage RNA polymerase is selected from the group consisting of T3, T7 and SP6, and a combination thereof.

252. (previously presented) The construct of claim 246, wherein said recognition site is a promoter for said RNA polymerase.

253. (previously presented) The construct of claim 245, wherein said nucleic acid produced from said construct is selected from the group consisting of DNA, RNA, a DNA-RNA hybrid and a DNA-RNA chimera, or a combination of the foregoing.

254. (previously presented) The construct of claim 253, wherein said DNA or RNA comprises sense or antisense, or both.

255. (currently amended) A nucleic acid construct which when introduced into a non-eukaryotic cell produces a ~~nucleic acid~~non-eukaryotic gene product comprising a ~~non-native~~eukaryotic intron, which when in a eukaryotic cell, said intron is ~~substantially~~ removed during processing and wherein said ~~nucleic acid~~gene product or protein expressed from a ~~nucleic acid~~gene product would be toxic specifically to a non-eukaryotic cell in the absence of said non-native intron.

Claims 256 and 257 (canceled).

258. (currently amended) The construct of claim 255, wherein said ~~nucleic acid~~ gene product is single stranded.

Claims 259-260 (canceled)

261. (currently amended) A nucleic acid construct which when introduced into a non-eukaryotic cell produces a ~~nucleic acid~~gene product comprising a non-native intron, wherein said product would be specifically toxic to a non-eukaryotic cell in the absence of said non-native intron and wherein said intron is ~~substantially~~ removed during processing and said intron is in a coding sequence of said ~~nucleic acid~~gene product.

REMARKS

Applicants again submit a paper and electronic copy of the Sequence Listing. Additionally, the specification has been amended to insert SEQ ID Nos. and to correct editorial errors which have heretofore gone unnoticed.

As will be discussed in further detail below, claims 245, 255, 258 and 261 have been amended to more distinctly claim that which Applicants regard as their invention and to advance prosecution. The claims amendments are supported by the specification. No new matter has been added.

1. The Rejections Under 35 U.S.C. 102 Over Wagner et al.

Claims 245-247 and 249-254 have been rejected under 35 U.S.C. 102(b) as being anticipated by Wagner et al, U.S. Pat. No. 5,591,601. The Office Action states that

> The claim limitation do not serve to free the claim from the prior art because the claim language does not require that the intron be a part of the polymerase, but rather requires only that the intron be contained on the nucleic acid[sic] construct.

The Office Action further states

> A broad reasonable interpretation of this language does not require the intron to be a part of the polymerase, but rather that an intron be contained somewhere on the construct as a whole. Because Wagner teaches a plasmid (i.e. a nucleic acid construct) that encodes two genes, the first being a gene of interest which includes a genomic gene and therefor contains introns non-native to the construct as a whole (e.g. col. 5, line 40-49), and the second being the non-eukaryotic polymerase T7 which acts to transcribe multiple copies of he gene of interest, Wagner is considered to teach all the instant claim limitations.

Applicants respectfully traverse the rejection. However as noted above, claim 245 has been amended to recite that the intron is in the coding

sequence of the polymerase. Given that the claim is directed to a nucleic acid construct the term "nonnative" does not have any significance. Applicants wish to emphasize that the amendment of claim 245 is not in acquiescence to the Examiner's position but to merely advance prosecution. The subject matter recited in amended claim 245 can thus be clearly distinguished from that disclosed in Wagner et al. The T7 polymerase does not contain a non-native intron. Applicants further note that 246-247 and 249-254 depend from claim 245 and would also thus not be anticipated by Wagner et al. in view of amended claim 245.

2. The Rejections Under 35 U.S.C. 102 Over Yamashita et al.

Claims 255 and 258-261 have been rejected under 35 U.S.C. 102(b) as being anticipated by Yamashita et al., 1990, Agric. Biol. Chem. 54:2801-9. The Office Action specifically states

> Yamashita et al. teaches a nucleic acid construct which produces a nucleic acid product comprising a non-native intron, which is removed during processing in a eukaryotic cell, and wherein said nucleic acid product or protein expressed from a nucleic acid product would be toxic to a non-eukaryotic cell in the absence of said non-native intron, wherein the nucleic acid product is single-stranded sense RNA...

Applicants respectfully traverse the rejection. However as noted above, claim 255 has been amended to recite that the claimed nucleic acid construct when introduced into a non-eukaryotic cell produces a **first non-eukaryotic gene product** comprising a eukaryotic intron which is removed during processing in a eukaryotic cell and produces a second gene product or protein; this second gene product or protein is toxic **specifically** to a non-eukaryotic cell in the absence of the intron. Applicants wish to emphasize that the amendment of claim 255 is not in acquiescence to the Examiner's position but to merely advance prosecution.

Claim 261 has been amended to recite that the nucleic acid construct when introduced into a non-eukaryotic cell produces a gene product comprising a **non-native** intron in the coding sequence of the gene product, wherein said product would be **specifically toxic** to a non-eukaryotic cell in the absence of said intron and wherein said intron is removed during processing.

In contrast, Yamashita et al. discloses the expression of human lymphotoxin gene containing SV40 introns in eukaryotic cells. Yamashita et al. can thus be distinguished from the subject matter recited in claims 255 and 261 in two respects. First, Yamashita et al. discloses the expression of **human lymphotoxin**, a eukaryotic gene; secondly, lymphotoxin produced in eukaryotic cells are actually thought to have more potential for therapy than lymphotoxin produced in noneukaryotic cells (see Yamashita et al., p. 2801, column 1, lines 9-15). Clearly, lymphotoxin is not specifically toxic to a non-eukaryotic cell in the absence of intron. Actually lymphotoxin is designed to be a therapeutic agent for various cancers. However, there is no evidence indicating that lymphotoxin is toxic to a non-eukaryotic cell in the absence of intron, since it is unlikely that a lymphokine would even be recognized by a non-eukaryotic cell.

For the reasons give above, the subject matter recited in amended claims 255 and 261 may be distinguished from Yamashita et al. Furthermore, claim 258 depends from claim 255 and would thus not be anticipated by Yamashita et al. Claims 259 and 260 have been cancelled.

Therefore, Applicants respectfully request that the rejections be withdrawn.

Summary and Conclusions

Claims 245-255 and 258-261 are presented for further examination. Claims 245, 255 and 261 have been amended.

No fee or fees are believed due for this paper or the accompanying Petition. In the event that any fee or fees are due, however, The U.S. Patent and Trademark Office is hereby authorized to charge the amount of any such fee to Deposit Account 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,

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MOLECULAR AND CELLULAR BIOLOGY, Apr. 1989, p. 1526–1535
0270-7306/89/041526-10$02.00/0
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Vol. 9, No. 4

Introduction of Functional Artificial Introns into the Naturally Intronless *ura4* Gene of *Schizosaccharomyces pombe*

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Received 17 October 1988/Accepted 15 December 1988

Insertion of a 36-base-pair (bp) synthetic oligonucleotide comprising the sequence 5'-GTAGGT(19N)CTAAT (4N)AG-3' into several different positions within the coding region of the naturally intronless *ura4* gene of *Schizosaccharomyces pombe* leads to an efficiently spliced gene producing a functional product. This suggests that the proper signals within an intron are sufficient to initiate and complete a splicing event independent of the location of the intron in the gene. Point mutations in the 5' junction (5'-GTAGGT-3') and in the putative branch sequence (5'-CTAAT-3') affect splicing efficiency significantly. A G-to-A transition at the first nucleotide at the 5' splice junction (5'-ATAGGT-3') abolishes the use of the authentic splice junction and leads to the increased use of an alternative splice site. No functional product is produced from this transcript. An A-to-G transition of the second A in the putative branch sequence (5'-CTAGT-3') lowers the splicing efficiency drastically, but still results in a functional gene product. Furthermore, extension of the 36-bp intron to introns more than 180 bp in size abolishes splicing, suggesting that the splicing apparatus might be restricted to very short introns. We discuss the possibility that *S. pombe* introns represent a simple type of eucaryotic intron.

Intervening sequences appear to be spliced from nuclear pre-mRNA by a common mechanism. The precursor RNA of eucaryotic cells is assembled into a spliceosome (splicing complex) in which the actual splicing event takes place (5, 7, 15, 23). Splicing can be viewed as a stepwise dynamic process which includes recognition of the intron, assembly of the spliceosome, and the actual splicing events (3, 13, 39). During this process, the precursor RNA is converted into an intermediate lariat structure by linking the first guanosine residue of the intron to an adenosine residue within the intron via a 2'-5' phosphodiester bond; the exon 1-intron junction is simultaneously cleaved. In the following steps, the 3' splice site is cleaved and exon 1 and exon 2 are linked together. The intron is released as a free lariat (9, 37, 42, 44).

There is evidence that at least five different small ribonucleoprotein particles (snRNPs) are involved in this process (29). These *trans*-acting factors are involved in the recognition of introns by interacting with specific *cis*-acting signals such as the 5' splice site, branch sequence, and 3' splice site of the precursor RNA (3, 4, 7). In addition, specific snRNP-snRNP interactions also seem to play a role in the spliceosome assembly and in the splicing process itself (4, 24). However, the precise functions of each of the snRNPs are not yet fully understood (6, 7, 27, 36). Single proteins are also involved in the splicing process, although little is known about their functions (28).

It is interesting that the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* appear to have introns which differ from one another and from those in higher eucaryotes. In contrast to the introns in the budding yeast *Saccharomyces cerevisiae*, all the introns found so far in *S. pombe* are very small. In *Saccharomyces cerevisiae*, intron sizes from 200 to 500 base pairs (bp) are predominant, whereas in *S. pombe* the size of the introns ranges from 36 to 121 bp (11, 17, 30, 48, 49). Also, the arrangement of the introns is very different. Almost all of the genes with introns in *Saccharomyces cerevisiae* contain one intron located very close to the beginning of the gene. In *S. pombe*, genes have been found containing 1 to 5 introns which are distributed throughout the coding region (11, 22, 30).

In contrast to *Saccharomyces cerevisiae*, the fission yeast *S. pombe* is able to splice the intron of the simian virus 40 small t antigen transcript correctly (21). A comparison of the intron of the small t antigen with all available *S. pombe* introns revealed an internal conserved sequence, 5'-CTPu APy-3', which was always found 3 to 16 nucleotides upstream of the 3' splice site (21, 30). Recently it has been shown that this sequence can serve in *S. pombe* as a branch sequence, forming a lariat with the A residue (30). In *Saccharomyces cerevisiae*, the highly conserved 5'-TAC TAAC-3' serves as a branch sequence (9, 26, 34). This sequence is found in all *Saccharomyces cerevisiae* introns and occurs 10 to 57 nucleotides upstream of the 3' splice site. The branch sequence in higher eucaryotes is quite variable, and the distance of the branch point from the 3' splice site is 10 to 40 nucleotides (16). In higher eucaryotes, a polypyrimidine tract between the branch sequence and 3' splice site plays an important role in splicing (13, 16, 43). In introns of *S. pombe*, the spacing between branch point and 3' splice is very small, indicating that most likely such a polypyrimidine tract does not play a role in splicing.

The 5' splice junction in *Saccharomyces cerevisiae* is, like the branch sequence, also highly conserved. In most cases, the sequence 5'-GTATGT-3' is found. In *S. pombe*, a consensus sequence, 5'-GTANTN-3', can be derived from the splice junctions known so far (30). This indicates that the 5' splice junctions of *S. pombe* are more variable than those of *Saccharomyces cerevisiae*. If we compare the 5' splice junctions of *S. pombe* with those found in higher eucaryotes, however, it appears that the 5' splice junctions in *S. pombe* do not display such a variability as has been shown for the 5' splice junctions in higher eucaryotes (30, 31).

When all of these observations are considered, it is evident that the architecture of introns, including the *cis*-acting splicing signals, has diverged. The introns found in *S. pombe* appear to represent a simple type of intron compared with the introns of *Saccharomyces cerevisiae* and those of

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higher eucaryotes. They are small and have no obligatory sequence conservation between the branch sequence and 3' splice site. We are interested in defining the nature and the degree of this evolutionary divergence as part of a general understanding of splicing in eucaryotes.

One of our goals is to identify mutations in *S. pombe* which affect mRNA splicing. A spliced gene whose expression is dependent on accurate removal of the intron and whose function can easily be monitored would be ideal for isolating suppressors. By selecting suppressors of mutations in the intron which abolish or lower the splicing efficiency, we should be able to identify *trans*-acting components of the splicing machinery (8). Either the known intron-containing genes from *S. pombe* are predominantly essential genes, or the introns are difficult to manipulate. In most cases, the introns are very small and lack convenient restriction sites for further manipulations such as intron extensions. Therefore, and particularly because we wanted a spliced gene with a biological phenotype, we inserted an artificial intron into the naturally intronless *ura4* gene from *S. pombe* (2). This gene encodes orotidine monophosphate decarboxylase, an enzyme of the uracil pathway.

Here we describe the construction of a *ura4* gene containing a 36-bp artificial intron whose function is dependent on a correct and efficient splicing event which can be monitored by selecting for uracil prototrophy among cells depleted for *ura4*. We introduced point mutations into the 5' splice junction and the putative branch point of the artificial intron. Analyses of the RNA produced in vivo from these mutated genes demonstrate that all the mutations affect splicing efficiency of the gene significantly. Interestingly, mutation of the first invariant G to an A in the 5' splice junction of the intron leads to the increased selection of an alternative splice site, while the authentic splice site is not used. Furthermore, intron extensions of more than 180 bp lead to the abolishment of splicing, suggesting that the splicing machinery in *S. pombe* is restricted to relatively small introns.

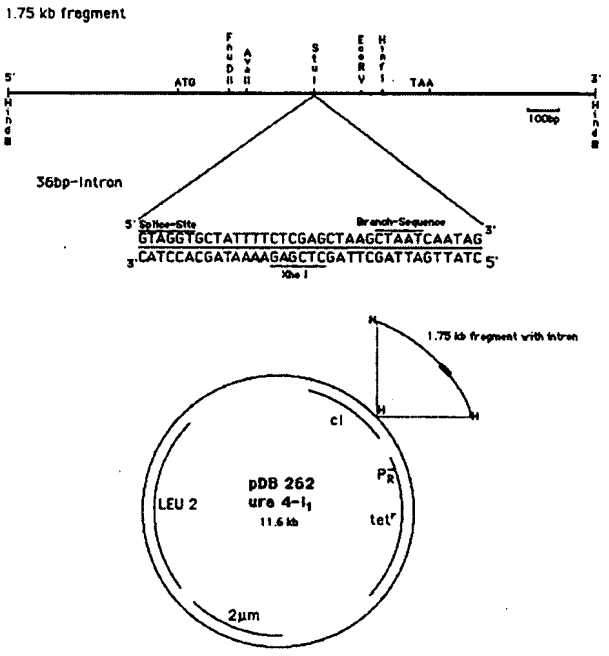


FIG. 1. Construction of the ura4-I_1 plasmid containing an artificial intron in the coding region of the *ura4* gene. A restriction map of the 1.75-kilobase-pair (kb) *Hin*dIII fragment carrying the *ura4* gene is shown. The location of the *ura4* coding region is indicated by the ATG start and TAA stop codons, respectively. The 36-bp double-stranded oligonucleotide has been inserted into the *Stu*I site as indicated. Putative 5' splice junction and branch sequence are overlined. The TAG within the putative 5' splice junction is an in-frame stop codon. The *Hin*dIII fragment containing this 36-bp insert (▬) has been cloned into the unique *Hin*dIII site of the shuttle vector pDB262, interrupting the *c*I lambda repressor gene. Inactivation of the repressor gene turns on the tetracycline resistance gene, allowing a positive selection for inserts (51).

MATERIALS AND METHODS

Yeast strains. *S. pombe* D18 (972 *leu1.32 ura4*) lacking the *ura4* gene, a gift from J. Kohli, was used as a recipient for the shuttle vector pDB262 (51) and all our constructions.

Construction of the pDB262 ura4-I_1 plasmid. The plasmid pDB262 ura4-I_1 (Fig. 1) was constructed as follows. A 1,750-bp *Hin*dIII fragment containing the *ura4* gene from *S. pombe* (2) was inserted into the *Hin*dIII site of a pUC8 plasmid. This plasmid was linearized with *Stu*I, which is a unique site within the coding region of the *ura4* gene at position 934 of the 1,750-bp *Hin*dIII fragment (Fig. 1). The 36-bp double-stranded blunt-ended oligonucleotide (Fig. 1) was inserted into this site, and the plasmid was transformed into *Escherichia coli* DH5 cells and selected for ampicillin resistance. We isolated several plasmids from different colonies and sequenced the double-stranded plasmids by using a 17-mer primer, 5'-GCAAGAGACCACGTCCC-3', which hybridizes to the coding region of the *ura4* gene 23 bp downstream of the *Stu*I site. The mini-prep method for isolating plasmids and sequencing by using a double-stranded template has been described earlier (14). We isolated the *Hin*dIII fragment from pUC8 plasmids containing the oligonucleotide in the direction indicated in Fig. 1 and inserted it into the *Hin*dIII site of the shuttle vector pDB262 containing parts of the 2µm DNA from *Saccharomyces cerevisiae* (51). The resulting plasmid, pDB262 ura4-I_1, was used to transform an *S. pombe* strain in which the *ura4* gene was deleted and which also contained the *leu1.32* mutation; thus, one could screen for *leu*$^+$ transformants because pDB262 contains the *LEU2* gene from *S. cerevisiae* which complements this mutation. For a functional test of the *ura4* gene containing the artificial intron, we could screen for uracil prototrophs. The *S. pombe* strain was transformed by using the lithium acetate procedure as described by Itoh et al. (19).

To test whether we could recover the plasmid ura4-I_1 from *S. pombe*, we isolated DNA by using the mini-prep method as described by Holm et al. (18). The isolated DNA was transformed into *E. coli* DH5 and screened for tetracycline-resistant colonies. Double-stranded sequencing of the recovered plasmids by using the method described earlier (14) confirmed that the ura4-I_1 construction was intact.

Construction of the intron extensions. The plasmid pDB262 ura4-I_1 contains a unique *Xho*I site which is located approximately in the middle of the 36-bp intron (Fig. 1). The plasmid was linearized with *Xho*I. DNAs from different sources were ligated into this site.

For extensions 108 and 196 (see Fig. 5), we used synthetic double-stranded oligonucleotides which were equipped with sequences to create *Xho*I overhangs. The oligonucleotide sequence used which appears in the transcribed RNA is 5'-TCGAGTCAAACGTCAATTAAAACGGAATATGTATCC CGGGATCCGTCGAGTACGAGTGTTCATGTTTCATGT

TTCAC-3'. Construction 196 contains this sequence as a tandem repeat. For extension 180 (see Fig. 5) we isolated a 150-bp *Xho*I-*Sal*I fragment containing sequences of the kanamycin resistance gene and some pBR322 sequences (32). For construction 252, we used φX174 sequences cut with *Hae*III, and for construction 350, pBR322 sequences cut with *Hae*III were used. In these cases, the plasmid was cut with *Xho*I and filled in with Klenow enzyme (New England BioLabs, Inc.).

After ligation, we transformed *E. coli* DH5 cells and isolated plasmids from different colonies by using the miniprep method as described previously (14). The length of the insertions was roughly estimated by cutting the plasmids with *Hin*dIII and running the digest on an agarose gel with the appropriate size marker. The sequences of the selected plasmids were verified by sequencing the double-stranded plasmids by using the primer described above (14).

Site-specific mutagenesis. The system used in these experiments was based on the method of Kunkel (25). The 1,750-bp fragment containing the *ura4* gene with the putative intron was ligated into the *Hin*dIII site of bacteriophage M13 mp19, and uracil-containing, single-stranded phage DNA was prepared. The following synthetic oligonucleotides were dephosphorylated and annealed to this single-stranded, uracil-containing phage DNA: 5'-AAATAGCACCTATCCT TGTATAATA-3' (mutation 5'-A1), 5'-AGAAAATAGCAC CGACCCTTGTATAA-3' (mutation 5'-3C), 5'-TCGAGAAA ATAGCCCCTACCCTTGTA-3' (mutation 5'-6G), and 5'-G AGGCTATTGACTAGCTTAGCTGAG-3' (mutation B-4G). For a summary of the mutant constructions, see Table 1. Double-stranded DNA was synthesized and ligated by incubating with Sequenase (United States Biochemical Corporation) and T4 ligase (Bethesda Research Laboratories, Inc.) for 2 h at 37°C under the conditions described by Kunkel (25).

After transformation of *E. coli*, white plaques were picked randomly and sequenced by the method of Sanger et al. (45) with a sequencing kit (United States Biochemical Corporation). The mutated genes were excised as 1,750-bp *Hin*dIII fragments from double-stranded phage and inserted into the shuttle vector pDB262 as described above. The double mutant 5'-A1/B-G4 was constructed by combining a *Hin*dIII-*Xho*I fragment containing the 5'-A1 mutation with a *Xho*I-*Hin*dIII fragment containing the B-G4 mutation and ligating it into pBD262. Exactly the same was done for the double mutation 5'-G6/B-G4. All these constructions were transformed into the *S. pombe* strain deleted for *ura4* and containing the *leu1.32* mutation by using the lithium acetate procedure (19).

RNA analyses. Total RNA was isolated from transformed cells as described previously (35). For S1 nuclease experiments, the double-stranded DNA probes were labeled either at the 5' end by using T4 polynucleotide kinase (New England BioLabs) or at the 3' end by using Klenow fragment (New England BioLabs). The labeled fragments were cleaved, and the appropriate singly end-labeled fragment was isolated. The S1 protection reactions were performed essentially as described previously (20). After the double-stranded DNA probes were denatured at 85°C for 15 min, DNA fragments were annealed to total RNA in 80% formamide–40 mM PIPES [piperazine-*N*-*N*'-bis (2-ethanesulfonic acid)] (pH 6.5)–400 mM NaCl–1 mM EDTA for 3 to 4 h at 52°C, lowering the incubation temperature by 1°C every hour.

Samples were treated with 150 U of S1 nuclease in 280 mM NaCl–30 mM sodium acetate (pH 4.5)–4.5 mM zinc acetate–20 μg of single-stranded DNA per ml for 30 min at 37°C. The fragments protected by RNA were precipitated and run on a sequencing gel as described in the figure legends.

To ensure that the S1 assays always contained the used probes in excess, the amount of probe added was determined by titration using a constant amount (60 μg) of RNA. To obtain complete digestion, the amount of S1 nuclease added was also determined by titration using a constant amount of RNA. The concentration of 150 U of S1 nuclease in our assay, as described above, was found to be appropriate.

Densitometric tracings. Autoradiographs of S1 experiments were scanned with a Soft Laser scanning densitometer (LKB Instruments, Inc.) by using the laser beam. Quantitation was done by cutting and weighing photocopies of the tracings.

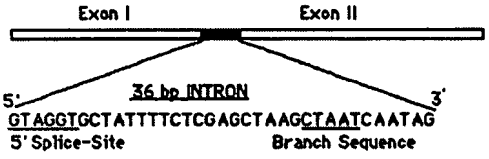
RESULTS

Construction of ura4-I_1, a *ura4* gene containing an artificial intron. The 1.75-kilobase-pair *Hin*dIII fragment containing the *ura4* gene (2) was used for the insertion of the double-stranded 36-bp oligonucleotide (Fig. 1). As possible 5' splice junction and putative branch sequence, we have chosen 5'-GTAGGT-3' and 5'-CTAAT-3', respectively. This 5' splice junction occurs in some natural introns of *S. pombe* (30). The sequence 5'-CTAAT-3' near the end of the intron has been found in several *S. pombe* introns (21, 30). The *Hin*dIII fragments containing the oligonucleotide in the indicated direction (Fig. 1) have been cloned into the yeast shuttle vector pDB262 (51). We refer to this construct as ura4-I_1 (Fig. 1). This construction was used to transform *S. pombe* D18, which is a *leu* mutant and is deleted for the complete *ura4* gene. We selected for *ura*$^+$ transformants. As a control, we also transformed into this strain a pDB262 plasmid carrying the natural *ura4* gene. The transformation efficiency for both of these plasmids was the same (Table 1). The construct ura4-I_1 complements the *ura4* deletion, demonstrating that this construct produces functional *ura4* enzyme. This indicates that a correct splicing event has occurred; a failure to splice should not lead to a functional enzyme, because of a stop codon in the artificial intron (Fig. 1).

S1 analyses of the RNA from ura4-I_1. To demonstrate that the transcripts from our construct ura4-I_1 were spliced correctly and to measure the splicing efficiency, S1 experiments were undertaken. To map the 5' splice site of the transcripts, a 3'-end-labeled 1,100-bp *Ava*II-*Hin*dIII fragment (Fig. 2, DNA probe a) was hybridized to total RNA isolated from cells containing the ura4-I_1 construction. After digestion with S1 nuclease, the remaining fragments were run on a sequencing gel. A 236-bp fragment should be protected from S1 digestion if the 5' splice site is used. The predominant band observed was at 236 bp (Fig. 2A, lane I_1), demonstrating the accurate use of the 5' splice site. We also detected a much less intense band at position 860, indicating the amount of mRNA precursor (Fig. 2A, lane I_1). The band at position 1100 indicates the reannealed probe in this experiment.

Relative proportions of mature RNA (Fig. 2, M) and pre-mRNA (Fig. 2, P) can be used as a measure of splicing efficiency. By densitometric scanning of the autoradiograph, we estimate that about 5% of the pre-RNA is unspliced. To demonstrate correct splicing of the transcripts at the 3' splice site, we performed S1 analysis using two fragments of different length labeled at the 5' end of the *Hin*fI site (Fig. 2, DNA probes b and c). In both cases, we detected an

TABLE 1. Summary of mutants and transformation efficiency of parental and mutant constructions[a]



		Designation	Transformation efficiency without supplementing uracil	Transformation efficiency supplementing uracil
ura4 without intron		ura4	+++	+++
GTAGGT	CTAAT	I_1	+++	+++
ATAGGT		5'-1A	-	+++
GTAGGG		5'-6G	++	+++
GTCGGT		5'-3C	-	+++
	CTAGT	B-4G	+	+++
ATAGGT	CTAGT	5'-1A/B-4G	-	+++
GTAGGG	CTAGT	5'-6G/B-4G	-	+++
Intron extension 108 bp		*108	+++	+++
Intron extension 180 bp		*180	++	+++
Intron extension 196 bp		*196	++	+++
Intron extension 252 bp		*252	-	+++
Intron extension 350 bp		*350	-	+++

[a] *S. pombe* D18 was transformed with 1 μg of parental or mutant plasmid (pDB262 containing inserts) and streaked out on minimal plates either without or with uracil (75 μg/ml). The colonies formed were counted: −, no colonies; +, <50; ++, <100; +++, <150 colonies per μg of plasmid DNA. Boldface letters indicate nucleotide changes.

S1-protected band at position 303, indicating that the correct 3′ splice site has been used (Fig. 2B, lanes I_1). The less intense bands seen at position 320 (first lane I_1) and at position 499 (second lane I_1) corresponded to the full-length protected probes. Although we are aware that these bands could be due to reannealing of the labeled probes, we are confident that these bands corresponded mostly to unspliced pre-mRNA. To determine the amount of reannealing, we hybridized the labeled probe (fragment *Hae*III-*Hin*fI) to total RNA isolated from cells which did not contain the ura4-I_1 construction; no reannealing could be detected (Fig. 2B, lane C).

These results clearly indicate that the construction ura4-I_1 displays a distinct biological phenotype which is associated with the removal of the inserted 36-bp oligonucleotide; therefore, this construction will be very useful for investigation of the effects of point mutations in the splicing signals. These experiments also demonstrate that sequences containing the proper 5′ and 3′ splicing signals are recognized and spliced out correctly independent of the surrounding sequence. This has been confirmed by inserting the same oligonucleotide into other locations, such as the *Eco*RV site of the *ura4* gene (Fig. 1). In this case, the intron was spliced out with the same efficiency; about 95% mature RNA could be detected (results not shown). It has been previously demonstrated that a synthetic intron is correctly spliced out when inserted into a rabbit globin gene, which contains several natural introns (40). It has also been demonstrated by using the β-globin gene that exon sequences can influence splice site selection and splicing efficiency of the gene (41). Our approach, inserting an artificial intron at various locations in a naturally intronless gene, shows that flanking regions of introns do not play an absolutely essential role in the recognition and correct splicing of introns. It appears in our case that splicing efficiency might be influenced by exon sequences to a minor extent, since efficiency is not 100%. This is probably due to secondary structure of the RNA which is not, however, a primary determinant of a precise splicing event (46).

Effect of point mutations in the 5′ splice junction and putative branch sequence of the ura4-I_1 construction. We used oligonucleotide-directed mutagenesis to change single nucleotides in the 5′ junction and in the putative branch sequence of our artificial intron in the ura4-I_1 construction. Single-nucleotide changes were made in the 5′ splice junction 5′-GTAGGT-3′ at three positions. We changed the first G to

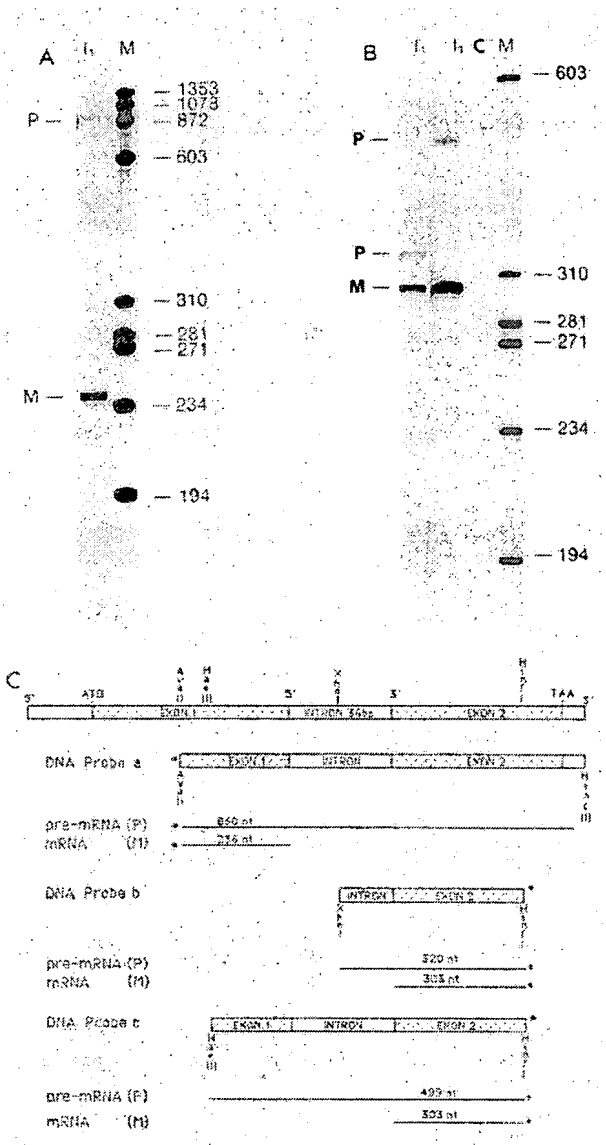


FIG. 2. S1 analyses determining the 5′ and 3′ splice sites of the transcripts from the ura4-I_1 construction. (A) To map the 5′ splice site, 30 μg of total RNA isolated from cells transformed with pDB262 ura4-I_1 was hybridized to DNA probe a, which is labeled at the 3′ end of the *Ava*II site (Fig. 2C, *) digested with S1 nuclease, and run on a 6% polyacrylamide–7 M urea gel. Lanes: I_1, RNA from cells transformed with pDB262 ura4-I_1; M, DNA size marker consisting of ϕX174, digested with *Hae*III and 5′ end labeled. (B) To map the 3′ splice site, 30 μg of RNA was hybridized to DNA probe b, which is labeled at the 5′ end of the *Hin*fI site (Fig. 2C, *), and to the DNA probe c, which is also labeled at the 5′ end of the *Hin*fI site (Fig. 2C, *). After digestion with S1 nuclease, the protected fragments were run on a 6% polyacrylamide–7 M urea gel. Lanes: I_1, RNA from cells transformed with pDB262 ura4-I_1; C, total RNA from cells transformed with the vector pDB262 (as a control for reannealing, probe c was hybridized to this RNA); M, ϕX174 DNA size marker. P, Precursor mRNA; M, mRNA; nt, nucleotides.

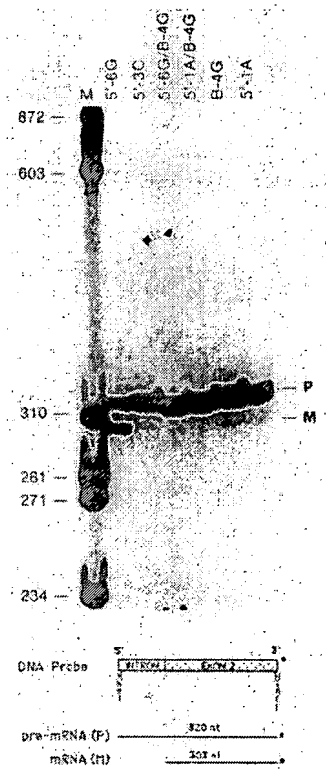


FIG. 3. S1 analyses: 3′ splice site determination of constructs containing point mutations in the 5′ splice junction and the branch sequence. Total RNA (60 μg) isolated from cells transformed with the indicated point mutations was hybridized to the 5′-end-labeled *Xho*I-*Hin*fI probe (*), S1 nuclease digested, and run on a 6% polyacrylamide–7 M urea sequencing gel. Lane M, ϕX174 size marker. P, Precursor mRNA; M, mRNA; nt, nucleotides.

an A, which we refer to as the 5′-1A mutation; the third A was changed to a C (5′-3C), and at the sixth position, a T was changed to a G (5′-6G). In the 5′-CTAAT-3′ putative branch sequence, we changed the fourth A to a G (B-4G). We also constructed the double mutations 5′-1A/B-4G and 5′-6G/B-4G, which contained both a nucleotide change in the 5′ junction and a change in the branch sequence at the indicated position.

First, we transformed the plasmids (pDB262) carrying these mutations into *S. pombe* D18 and selected for *ura*$^+$ transformants. In a second transformation experiment, we selected for *leu*$^+$ transformants on medium supplemented with uracil. Table 1 (lanes 2 and 3) shows the transformation efficiencies of the mutations as compared with the transformation efficiencies of the ura4-I_1 construction and the natural *ura4* gene. While both the 5′-6G and the B-4G mutations gave rise to uracil prototrophs, the transformation efficiency of the mutation B-4G was very low. All the other mutants (Table 1) did not form colonies when selected for *ura*$^+$ transformants.

For further analyses, we isolated total RNA from cells carrying these mutations, selected for leucine prototrophy. S1 analyses were performed by using the 5′-end-labeled probe (fragment *Hin*fI-*Xho*I) shown in Fig. 3. This probe maps the 3′ splice sites of the constructs. The relative proportions of mature RNA (Fig. 3, M) and pre-mRNA (Fig. 3, P) are an indicator of splicing efficiency. As can be seen in

Fig. 3, in all cases, except for the mutation 5'-6G, pre-mRNA accumulated and less than 1% mature RNA (M) could be detected. This indicates a strong inhibition of splicing. The 5'-6G mutation also affected splicing efficiency, but about 50% mature message could be detected (Fig. 3, lane 5'-6G), which apparently provided sufficient translatable message to render the cells prototrophic for uracil. The same was true for the mutation B-4G, although the level of mature message was not much higher than the level of mature RNA in the mutations which did not confer uracil prototrophy (Fig. 3, lane B-4G). This was somewhat surprising. It was anticipated that particularly the 5'-1A mutation and the B-4G mutation would not produce mature message. It has been reported for the yeast *Saccharomyces cerevisiae* and for higher eucaryotes that changes of the invariant first G of the 5' splice junction lead to the accumulation of lariat structures which are not further processed (12, 34, 46). Mutations of the first nucleotide of the 5' splice junction in *Saccharomyces cerevisiae* clearly lead in vivo to the accumulation of splicing intermediates, and no mature message can be detected (12, 34). For mutations in the highly conserved branch sequence 5'-TACTAAC-3', conflicting results have been reported. In *Saccharomyces cerevisiae*, when the A at position six is mutated, splicing is not completely prevented (12, 50). This A has been shown to be the branch point to which the first G of the 5' splice junction is linked to form a lariat (9). The B-4G (A→G) mutation in the 5'-CTAAT-3' sequence of our intron also did not prevent splicing; however, splicing efficiency was drastically reduced, although cells containing this mutation were able to grow without supplementing uracil. Mertins and Gallwitz (30) have shown that this A within this sequence in *S. pombe* introns is the branch point.

We do not yet know whether this A actually serves as a branch point, nor do we have enough data to characterize exactly the structure of a branch sequence, but it seems clear that in *S. pombe*, the branch sequence conservation is critical, as it is in *Saccharomyces cerevisiae*; otherwise, we would not expect such a drastic reduction in splicing efficiency. There are still several branchlike sequences in that region containing an A to compensate, if the branch sequence could vary as widely as in higher eucaryotes (Fig. 1; 36, 46).

We mapped the 5' splice sites of the mutations by S1 analysis using the 3'-end-labeled *Ava*II-*Hin*dIII probe shown in Fig. 4. It is important that for each mutation, the *Ava*II-*Hin*dIII fragment from the mutant allele was used as a probe. As can be seen in Fig. 4, the major protected band could be detected at position 860, which is the size of the pre-mRNA (Fig. 4, P). The less-intense band in this area, which was somewhat smaller than 860 nucleotides, was probably due to a second transcriptional stop site of the *ura4* gene. In the strains carrying the B-4G, 5'-3C, 5'-6G, and 5'-6G/B-4G mutations, a less-intense band at position 236 could be observed. This band corresponded to the mature message (Fig. 4, M). In addition, we observed a very faint band at position 223. This RNA species (Fig. 4, C) was also detected in the cells containing the ura4-I_1 construction (Fig. 4, lane I1). However, we could detect this RNA species only when we used increased amounts of RNA (60 μg) and when we overexposed the film. Interestingly, the mutations 5'-1A and 5'-1A/B-4G produced a significantly higher amount of this smaller RNA species (C), whereas no mature message (M) could be detected in these mutants (Fig. 4, lanes 5'-A1 and 5'-A1/B-4G). These results suggest that an alternative 5' splice site was used, which is located 13 nucleotides upstream of the authentic 5' splice junction. Indeed, in this area we found a sequence, 5'-G/GTATTA-3', which resembled the consensus sequence 5'-G/GTANGN-3' for *S. pombe*, although the T at the second-to-last position of this alternative site was a clear mismatch (30). That may explain why this 5' splice site was used so inefficiently. When the invariant G of the authentic splice site was mutated to an A (5'-A1), the efficiency with which this alternative splice site was used improved markedly.

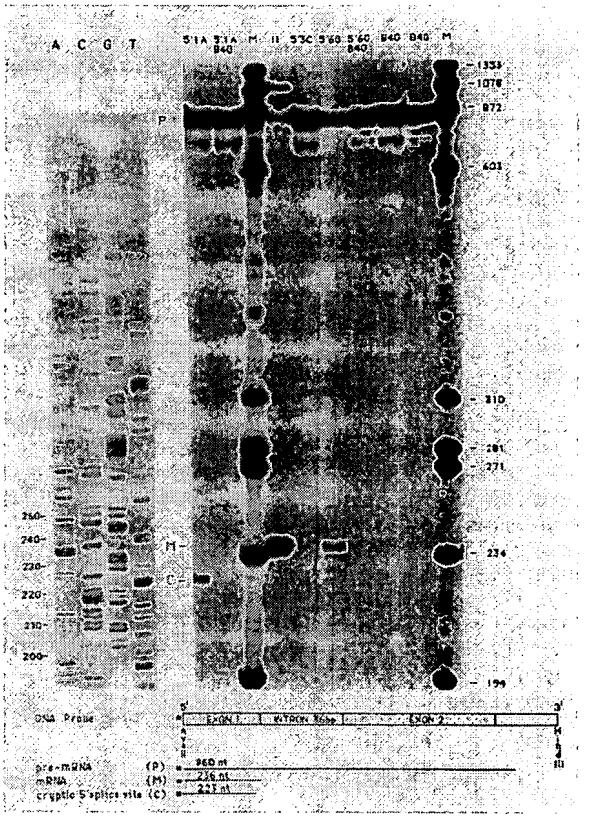


FIG. 4. S1 analyses: 5' splice determination of constructs containing point mutations in the 5' splice junction and the branch sequence. Total RNA (60 μg) isolated from cells transformed with the indicated point mutations was hybridized to the 3'-end-labeled *Ava*II-*Hin*dIII probes (*), S1 nuclease digested, and run on a 6% polyacrylamide–7 M urea sequencing gel. Lanes: I1, total RNA from cells transformed with pDB262 ura4-I_1; M, φX174 size marker. A,C,G,T is the sequence pattern of a known sequence used as a size marker. P, Precursor mRNA; M, mRNA; C, alternative splice site; nt, nucleotides.

To analyze whether the mutations 5'-1A and B-4G accumulate splicing intermediates such as lariat structures, as reported for the yeast *Saccharomyces cerevisiae* (12, 34, 50), we performed primer extension analyses using a 17-nucleotide synthetic primer which hybridizes in exon 2, 20 nucleotides downstream of the 3' splice site. In both cases, we detected accumulation of pre-mRNA; however, we could not detect a primer extension product which mapped to the branch point, which would indicate accumulation of lariat structures (results not shown). In higher eucaryotes and *S. pombe*, lariat structures are very unstable in vivo, which may account for our inability to detect these structures (30, 52). However, it is remarkable that in the 5'-1A mutation the use of the alternative splice site is increased; although this

site is only a weak match to the consensus sequence, it has a G at the first position, suggesting that in *S. pombe*, a G at this position is necessary to allow the formation of the lariat. The 5'-3C mutation affects splicing as severely as the 5'-1A mutation, but the authentic 5' splice is still used. This clearly indicates a functional difference between the nucleotides in these two positions.

Effect of intron extensions of the artificial intron. To test whether extensions of the 36-bp intron in the ura4-I_1 construction have a significant effect on splicing efficiency, we inserted DNA of different lengths and from different sources into the *Xho*I site of the artificial intron (Fig. 1 and Materials and Methods). The resulting plasmids were isolated and identified by double-stranded sequencing using a 17-mer primer downstream of the 3' splice site of the intron.

Plasmids containing introns with lengths of 108, 180, 196, 252, and 350 bp were constructed and transformed into *S. pombe* D18. First we selected for *ura*$^+$ transformants. Under these selection conditions, only the constructions containing introns of 108, 180, and 196 bp formed colonies. The constructions containing 252- or 350-bp introns did not form colonies (Table 1). This indicates that these introns are spliced out inefficiently, if at all.

To measure the influence of intron length on splicing efficiency, we performed S1 analysis to determine the relative proportions of mature RNA and pre-mRNA as described above. We isolated total RNA from cells transformed with the constructions indicated above in which selection was for *leu*$^+$ and uracil was supplemented. The 5'-end-labeled probe shown in Fig. 5 was hybridized to total RNA and digested with S1 nuclease, and the products were run on a denaturing gel.

As can be seen in Fig. 5, only the constructs containing introns of 108, 180, and 196 bp show a significant band at position 303, which is the expected size for the mature RNA (Fig. 5, M). Constructs containing the introns 252 bp and 350 bp in length do not show significant amounts of mRNA (M). Predominantly pre-mRNA (Fig. 5, P) can be detected at position 320. These results are consistent with the transformation results described above.

The constructions containing the longer introns do not enable the cells to grow without uracil. Depending on the length of the intron we observe a difference in the relative proportions of mature RNA (M) and pre-mRNA (P). The constructions containing 180- and 192-bp introns show a higher amount of pre-mRNA (P) compared with mature RNA (M) than the constructions containing the 108-bp intron and our construction ura4-I_1, containing the 36-bp intron (Fig. 5). Additional intron extension constructions have been tested. The ratio of precursor RNA (P) to mature RNA (M) always increases significantly when the intron is longer than 180 bp. Introns larger than 220 bp did not splice well, if at all (results not shown). Although this inhibitory effect, which is dependent upon a certain length of the intron, might be due to the negative effect of secondary structures of the splicing substrate, we do not think that this is the only explanation for this effect. It is important that all natural introns in *S. pombe* found so far are very short, with the longest intron being 120 bp (30, 48, 49). The architecture of our artificial intron is typical of introns occurring naturally in *S. pombe*. Parker and Patterson described this type of fungal intron as 5'S/3'S, characterized by a short distance between the 5' splice site and the branch point and by a short distance between the branch point and the 3' splice site (38). All natural introns of *S. pombe* without exception so far conform to this description. In this light, it is interesting that

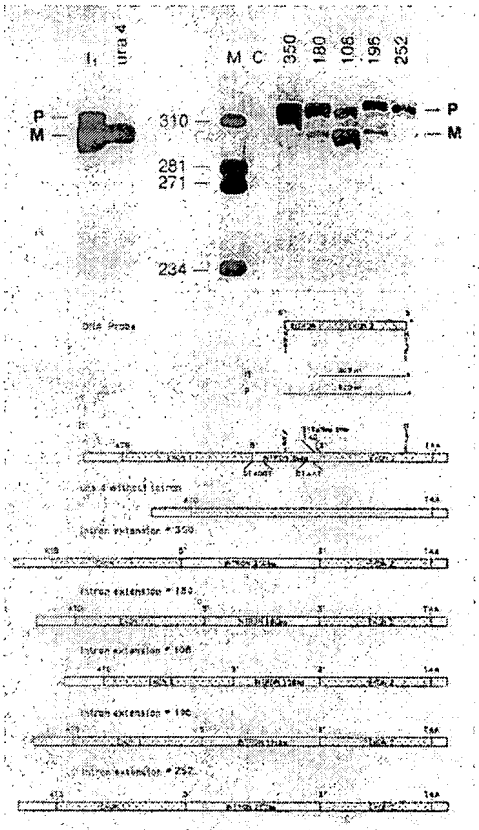


FIG. 5. S1 analyses to determine the 3' splice sites of ura4-I_1 constructions containing artificial introns of different lengths. Total RNA (60 μg) isolated from cells transformed with the indicated intron extensions (in base pairs) was hybridized to the 5'-end-labeled *Xho*I-*Hin*fI probes (*), S1 nuclease digested, and run on a 6% polyacrylamide–7 M urea sequencing gel. Lanes: I_1, RNA isolated from cells transformed with the ura4-I_1 construction; ura4, RNA from cells transformed with a plasmid containing the natural *ura4* gene; M, ϕX174 size marker; C, RNA from a cell transformed with the plasmid pDB262, as a control of reannealing of the probe in these experiments. P, Precursor mRNA; M, mRNA; nt, nucleotides.

Parker and Patterson speculate that this class of introns, in its simplicity, might be the progenitor of the more sophisticated introns, which are longer and have an extended distance between branch point and 3' splice site, including the pyrimidine tract in higher eucaryotes (38). On the basis of our results described above, and taking into consideration that until now only 5'S/3'S introns have been detected in *S. pombe*, it is conceivable that the splicing machinery in *S. pombe* might be restricted to introns of this type.

We have some indirect evidence in support of this hypothesis. The large intron (347 nucleotides) of the simian virus 40 large T antigen is not spliced in *S. pombe*, although the 5' splice junction conforms to the 5' splice site consensus in *S. pombe*, as does the 5' splice of the small t antigen intron, which is spliced perfectly (21). The small intron of the early region of simian virus 40 is clearly a 5'S/3'S intron. All our other attempts to splice foreign introns in *S. pombe* failed. None of the introns which we used resembled the 5'S/3'S type (results not shown).

DISCUSSION

In this report, we have demonstrated that the *cis*-acting signals within an intron are sufficient to specify intron excision in a novel exon environment. The exon-intron boundaries which have been created are GG/GT or AT/GT. A survey of exon-intron boundaries of naturally occurring introns in *S. pombe* revealed the following boundaries: AA/GT, AG/GT, CG/GT, CT/GT, TG/GT, and TT/GT (17, 30, 48, 49); thus, we have created exon-intron boundaries which have not yet been found in natural *S. pombe* genes, yet splicing is correct and efficient. We note that similar experiments have been reported for inserting a synthetic intron into a rabbit β-globin gene (40). However, the basis on which a splice junction is primarily selected remains controversial. It is interesting that in the yeast *Saccharomyces cerevisiae*, exon 1 sequences are obviously not needed to align the 5′ splice sequence with the snRNP U1 for spliceosome assembly (10). Although it has been shown that the 5′ splice junction base pairs with the 5′ end of U1 snRNPs (33, 53) and that 5′ splice junctions with better matches might be selectively utilized, it has been demonstrated that the context of the 5′ splice site influences the selection (1). Our results indicate that splice site selection in *S. pombe* may occur largely independently from the surrounding exons. The 5′ splice site chosen under competitive conditions was the best overall match to the consensus sequence. Although the authentic splice site of the artificial intron is predominantly used, a sequence with a weak match to the 5′ splice consensus sequence of *S. pombe* which appears 13 nucleotides upstream of the authentic splice site is also used (Fig. 4, lane I1).

For *S. pombe*, it has not been shown experimentally that a U1 snRNP interacts with the 5′ splice junction, although the U1 molecule from *S. pombe* contains sequences at the 5′ end which are complementary to the 5′ splice consensus sequence of *S. pombe* (J. A. Wise, personal communication). Our ura4-I_1 construction containing the artificial intron provides a useful tool to further investigate splice site selection within a novel RNA environment.

Our point mutations within the 5′ splice junction and the branch sequence clearly demonstrate that single changes within these conserved regions significantly affect splicing efficiency. However, depending on the base position within the 5′ splice junction, splicing efficiency is affected to a different degree. Most interestingly, the transition mutation G→A of the first intron nucleotide leads to a complete shift of splice sites. The authentic splice site is not used at all, whereas the alternative splice site is now used more efficiently. Such an effect has not been found in *Saccharomyces cerevisiae*. In *Saccharomyces cerevisiae*, such G-to-A mutations allow the formation of the intermediate lariat structure, which is not further processed (12, 34, 46). We could not detect accumulating lariat structures. Instead, an alternative 5′ splice has been chosen which conforms only weakly to the consensus 5′ splice site found for *S. pombe*, but it has a G as its first nucleotide. This observation, taken together with the fact that the 5′-3C mutation affects splicing efficiency as severely as the 5′-A1 mutation while the 5′-3C mutation still uses its authentic splice site (Fig. 4, lane 5′3C), suggests that in *S. pombe* a G in the first position is not only necessary but absolutely required for forming an intermediate lariat. However, further experiments, particularly in an in vitro *S. pombe* splicing system, are necessary to confirm this observation.

As discussed before, the architecture of introns found so far in *S. pombe* might reflect the simplest type (5′S/3′S) of pre-mRNA introns (38). They are small, and specific sequences between the branch point and the 3′ splice site seem not to have a function for determining a precise splicing event. It is interesting that all of our intron extensions over 180 bp led to a significant decrease or abolishment of splicing, although these introns are still very small compared with naturally occurring introns in *Saccharomyces cerevisiae* and higher eucaryotes (11, 46).

On the basis of our experiments, we cannot completely exclude that these longer introns fail to be spliced out because of unfavorable secondary structures. However, since we have tested a large number of extensions by using DNA from different sources, it is highly improbable that structural features alone account for this drastic effect. Furthermore, some of the sequences used also have been inserted into *Saccharomyces cerevisiae* introns without any effect on splicing efficiency (47). In *S. pombe* it is conceivable that, because of the short distance between the branch point and the 3′ splice site, there is no requirement for a component which might have a function in positioning other components to make intron ends meet. From this it would follow that only short introns can be spliced in *S. pombe*. This hypothesis would contrast to the situation for *Saccharomyces cerevisiae* and higher eucaryotes, where the sequences between branch point and 3′ splice site are necessary for the binding of spliceosome components (3, 6, 13, 27, 43).

Finally, we have developed a powerful selection system for suppressors of splicing defects. Cells containing our construction ura4-I_1 can grow without supplementing uracil only if the 36-bp artificial intron is accurately and efficiently spliced. As discussed above, all of our point mutations introduced either in the 5′ splice site or in the branch sequence cause a significant decrease in splicing efficiency which leads to growth defects or complete growth inhibition. We are now in the position to screen for extragenic suppressors that restore the splicing efficiency of these mutations. A similar approach has been used to identify a gene in *Saccharomyces cerevisiae* whose product probably interacts with the branch sequence (8).

ACKNOWLEDGMENTS

We are grateful to Horst Domdey for providing the oligonucleotides used for creating the artificial intron and to Paul Ryan for the preparation of the oligonucleotides used for mutagenesis. We thank J. Kohli for providing *S. pombe* D18 and the plasmid containing the *ura4* gene.

We thank the DAAD (Deutscher Akademischer Austauschdienst) for fellowships awarded to A. Hoffmann and K. B. Gatermann. This work was supported by a Drexel Mini Grant, by a Biomedical Research Support grant, and by a grant (MV 389) from the American Cancer Society to N. F. Käufer.

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imity to the blood stream endothelial cells are an obvious candidate for delivering therapeutic proteins systemically. Genetically engineered vascular grafts could also be used to target the delivery of a therapeutic protein to a specific organ or limb that is perfused by blood passing through the grafted artery. Specific applications might include the secretion of vasodilators or angiogenic factors to ischemic myocardium or the delivery of an antineoplastic agent to an organ riddled with metastatic tumor.

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5. The BAG vector, which expresses β-galactosidase from a transcript initiated at the 5′ long terminal repeat (LTR), has been described (*4*). The BAL vector is derived from the previously described BA-LDLR vector [J. M. Wilson, D. E. Johnston, D. M. Jefferson, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4421 (1988)], except that low density lipoprotein receptor (LDLR) cDNA sequences were replaced with coding sequences for the *lacZ* gene. Expression of *lacZ* from this vector is driven from a transcript that it initiated at β-actin promoter sequences located internal to the viral transcript. Each vector was transfected into the amphotropic packaging cell line ψ CRIP [O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988)], and individual transfectants were analyzed for production of recombinant virus. Viral titer was measured by exposing subconfluent plates of NIH 3T3 cells to limiting dilutions of viral stocks and subsequently analyzing the confluent fibroblasts for clones of β-galactosidase–expressing cells (*4*). Titers of the best virus-producing cell lines were 0.5×10^5 to 1×10^5 colony-forming units (CFU) per milliliter for BAG-derived virus and 2×10^5 to 5×10^5 CFU per milliliter for BAL-derived virus. Virus-producer cell lines and infected populations of endothelial cells were free of helper virus.
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8. We thank M. Grossman and J. Hoysradt for technical assistance, and K. Gould and R. Connolly for helpful advice. Supported by the NIH, Whittaker Foundation, and Howard Hughes Medical Institute.

19 January 1989; accepted 28 April 1989

Control of Gene Expression by Artificial Introns in *Saccharomyces cerevisiae*

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Artificial yeast introns that show cold-sensitive splicing have been constructed. These conditional introns can be inserted into a target gene as an "intron cassette" without disrupting the coding information, allowing expression of the gene to be cold sensitive. Insertion of these intron cassettes rendered the yeast *URA3* gene cold sensitive in its expression. The advantage of this intron-mediated control system is that any gene can be converted to a controllable gene by simple insertion of an intron.

ONLY A SMALL NUMBER OF GENES in *Saccharomyces cerevisiae* contain introns, which are usually found individually near the 5′ end of their genes (*1*). The role of such rare introns is not clear. The removal of an intron from the yeast actin gene did not affect its expression (*2*). Nevertheless, they do appear to have a regulatory function in some cases. For example, splicing of an intron in the yeast ribosomal gene *RPL32* is autogenously controlled by its product (*3*).

Each yeast intron contains three conserved sequences, GUAPyGU (Py, pyrimidine) at the 5′ donor end, UACUAAC at the branch point, and PyAG at the 3′ acceptor end (*4*). Base pairing between small nuclear RNAs (snRNAs) and GUAPyGU and UACUAAC sequences is thought to be important for efficient splicing (*5*). These consensus sequences are similar to those found in higher eukaryotes. However, the sequence requirement is more stringent in yeast than in higher eukaryotes (*6*). In addition, there are no conserved sequences in the flanking exons of yeast.

Heterologous introns have been successfully inserted into target genes in order to study mRNA splicing (*2, 3, 5, 7, 8*). The same strategy was also used to study Ty transposition (*9*) and activation at upstream sites (*10*). However, in these investigations, an intron was inserted along with its flanking exon sequences. These flanking sequences can cause inactivation of the target gene. Therefore, we have inserted an artificial intron totally devoid of flanking exon sequences and have used it to control the heterologous gene expression.

The intron consisted of 30 bp of the 5′ end of the yeast *RP51A* intron (*7*), 30 bp of the 3′ end of the yeast *S10* intron (*11*), and 44 bp of polylinker sequence of an M13 cloning vector (Fig. 1). This intron contains all the necessary sequences for splicing in yeast. In addition, we have placed Sna BI and Pvu II restriction sites at the ends. Since the Sna BI and Pvu II recognition sequences overlap with the three terminal bases at each intron end, the artificial intron can be cleaved out precisely as a "cassette" without any additional exon sequences. This intron fragment can be inserted into any gene without disrupting its coding information by cutting the gene with a blunt end–producing restriction enzyme and then inserting the intron fragment. Alternatively, if the restriction enzyme leaves a protruding 5′ terminus, the fragment can be treated at one end with single strand–specific nuclease and at the other end by the Klenow fragment of DNA polymerase I.

To show that the artificial intron can be inserted into a gene without disrupting its function, we inserted it into the *URA3* coding region at Eco RV, Sca I, or Stu I sites (*12*), which are located 186, 310, and 434 bp downstream of the start codon, respectively. These intron-containing *URA3* genes were located on a "2μ plasmid" construct (*13*). This construct was able to complement a *ura3-52* mutation on the yeast chromosome (*14*), which suggests that the artificial intron is spliced out precisely and

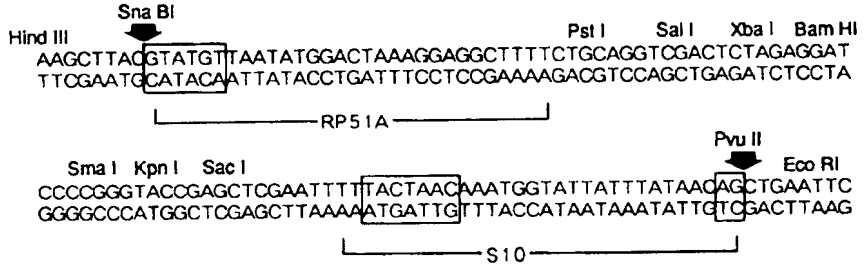

Fig. 1. Structure of the artificial intron. The 5′ end fragment and the 3′ end fragment were chemically synthesized, and the polylinker fragment (from Pst I to Sac I) was obtained from plasmid pUC18. The consensus sequences are shown in boxes. This intron was cloned in the Hind III–Eco RI site of pUC18 to generate pUC-AI. The 5′ end fragment of the yeast *RP51A* intron and the 3′ end fragment of the *S10* intron are indicated.

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Table 1. β-Galactosidase activity of the *PGK-lacZ* fusion gene with an intron cassette. See (*15–17, 21*) for details of the construction of the *PGK-lacZ* gene with and without the modified introns. Cells containing CEN plasmids were grown in minimal medium SD (*23*) supplemented with histidine and leucine for at least 20 generations at the indicated temperature. Assay for the β-galactosidase was performed as described (*24*). Each value represents the average of four experiments and the error was less than 30%. AI, artificial intron; AI-1 and AI-2, AI with inverted repeats; AI-3 and AI-4, cold-sensitive introns.

Construct	β-Galactosidase activity (units/mg protein)		
	36°C	23°C	16°C
None	750	860	1190
AI	870	920	1150
AI-1	7	6	8
AI-2	8	5	6
AI-3	120	21	11
AI-4	200	54	23

further implies that the artificial intron can be inserted anywhere in a target gene without disrupting its function.

To determine the efficiency and the accuracy of splicing, we inserted this intron into the *PGK-lacZ* fusion gene (*15*). The β-galactosidase activity produced by the intron-containing gene (*PGK-lacZ-AI*) was about the same as that produced by the intronless *PGK-lacZ* gene (Table 1). The level of mRNA produced by the *PGK-lacZ-AI* gene was also about the same, and a majority of the *lacZ* transcript was of the mature form (Fig. 2A), indicating that splicing of the artificial intron was efficient and accurate.

Base pairing between consensus sequences of an intron and snRNAs is important for the splicing reaction (*5*). Accordingly, we thought that introduction of a fragment complementary to one of the consensus sequences would be likely to interfere with this base pairing and thus prevent splicing. If so, by varying the length of the complementary fragments, we might be able to obtain a fragment that interferes with splicing at a low temperature but not at a high temperature. Splicing of the intron containing such a fragment would then become cold-sensitive.

To see the effect of an inserted fragment that can base pair with one of the consensus sequences, we have added a fragment that contained either the 5′ donor consensus sequence (GUAUGU) or the branch point sequence (UACUAAC) into the artificial intron in the inverted orientation. These two constructs contain 33-bp (*AI-1*) and 38-bp (*AI-2*) inverted repeats, respectively (*16, 17*). Yeast were transformed with plasmids containing the *PGK-lacZ* fusion gene plus one of these modified introns, and the level of β-galactosidase activity was measured. The level of β-galactosidase activity was about 1/100 of that of the parent (Table 1). Mature mRNA was not detected by primer extension (*14*). These results indicate that splicing is inhibited by an inverted repeat structure at the 5′ consensus region or the UACUAAC region. The inverted repeat structure itself was not responsible for this inhibition, since unrelated inverted repeats in the intron did not prevent splicing (*14*).

To obtain cold-sensitive introns, we made Bal 31 deletions of the *AI-1* and *AI-2* introns, starting either from the center of the inverted repeat (for *AI-1*) (*18*) or from the outside of the inverted repeat (for *AI-2*) (*19*). Introns with various sizes of inverted repeats were isolated and then inserted into the *PGK-lacZ* fusion gene. We looked for cold-sensitive introns by growing each yeast transformant on X-gal plates at either 36°C or 16°C (*20*). If the introns were spliced out, the cells would produce active β-galactosidase and form blue colonies; otherwise, white colonies would be observed. We found two deleted introns that led to the formation of blue colonies at 36°C and white colonies at 16°C, one from *AI-1*, the other from *AI-2*. We call these cold-sensitive introns *AI-3* (13-bp inverted repeat) and *AI-4* (17-bp inverted repeat), respectively (*21*). The β-galactosidase activities produced by these genes (Table 1) were clearly heat inducible, although even at the highest temperature the activities were 15 to 20% of those produced by the intronless gene. Analysis of the mRNAs by primer extension showed that these genes produce the mature size of mRNA at 36°C but not at 16°C (Fig. 2A). The possible RNA secondary structures of these introns are shown in Fig. 2B. In both cases the consensus sequence, GUAUGU or UACUAAC, is in a short hairpin structure. These short hairpin structures may be formed at 16°C (preventing splicing of the intron), but melt at 36°C (allowing the intron to be spliced out) in vivo. We have not ruled out the possibility that other cellular factors are involved.

We wanted to show that insertion of the

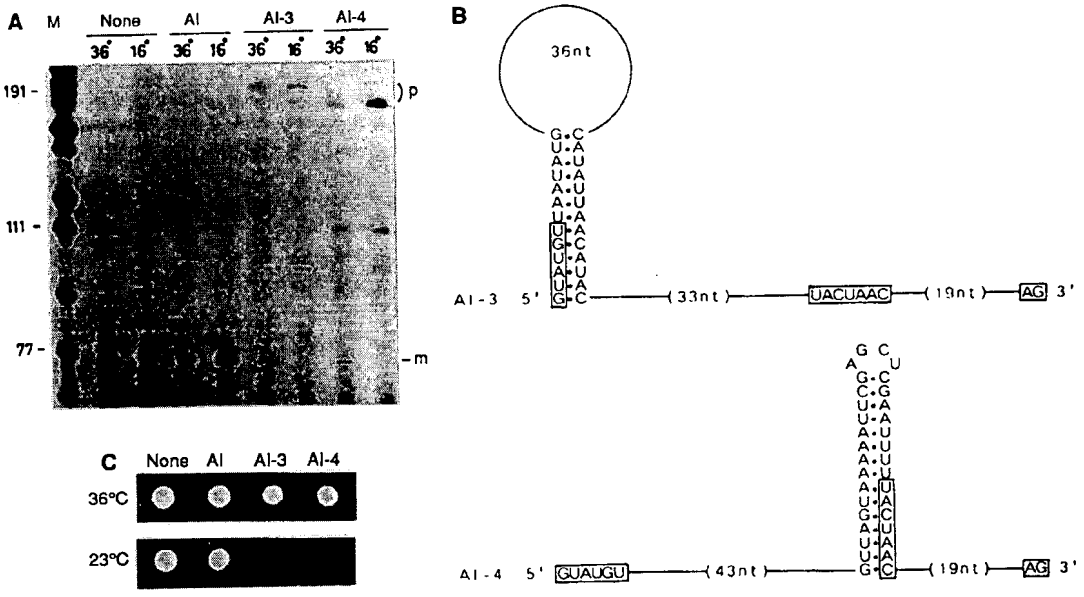

Fig. 2. The cold-sensitive introns. (**A**) Analysis of *PGK-lacZ* mRNA by primer extension. Cells were grown as described in Table 1. Total RNA was extracted and primer extension was performed as described (*25*). M13 sequencing primer [a 15-nucleotide oligomer (New England Biolabs)] was used as a primer. Precursor RNAs are indicated by p. Mature mRNA is indicated by m. pBR322 DNA cut with Hpa II and labeled with [α-^{32}P]dCTP and Klenow enzyme was used as a size marker (lane M). (**B**) Possible structure of the cold-sensitive introns. (**C**) Phenotype of the yeast strains NY6A (*MATa, ura3-52, leu2-3, leu2-112, his4-519*) containing the *URA3* gene with the *AI-3* or *AI-4* intron on 2μ plasmid (*13*). Cells were grown overnight at 23°C in minimal medium SD (*23*) supplemented with uracil and histidine, then spotted on SD plates supplemented with histidine (about 5000 cells in each spot). Plates were then incubated either at 36° or 23°C for 3 days.

AI-3 and *AI-4* intron could render other target genes cold-sensitive as well. We have inserted these cold-sensitive introns into the Stu I site of the yeast *URA3* gene (*URA3-AI3*, *URA3-AI4*). As expected, the yeast strain having either the *URA3-AI3* or the *URA3-AI4* gene on a 2μ plasmid showed a cold-sensitive URA phenotype (Fig. 2C). It is not likely that cold sensitivity is due to the genetic background, since experiments with another nonisogenic strain gave the same results (*14*).

In this report we have described an approach to the control of gene expression by an artificial cold-sensitive intron. This approach can be applied to foreign gene expression driven by a strong promoter whose expression is difficult to control. Further modification of the artificial intron will be necessary, however, to obtain optimal expression in the induced state and no expression at the low temperature.

This intron makes it possible for investigators to disrupt gene function conditionally, which will be helpful in studying the function of essential genes and in isolating mutants. For mutagenesis, it may be necessary to mark the cold-sensitive intron with a genetic marker.

In addition, the artificial intron can be modified in many other ways. For example, an operator sequence could be inserted to control transcription of a target gene by binding of a repressor. A native intron that has self-regulated splicing can also be used. Such a native intron cassette can be easily obtained by amplifying only the intron sequence by polymerase chain reaction (PCR) methods (*22*). This type of native intron cassette as well as artificial introns would allow us to control any gene in yeast in a variety of ways.

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13. The 2μ plasmids were constructed as follows: An artificial intron was inserted into *URA3* on plasmid YIp5. An Sph I–Pvu II fragment containing *URA3* was released from the plasmid, then cloned into the Sph I–Pvu II site of YEp13, which contains the 2μ origin and *LEU2*.
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15. The Mbo II cleavage site at the initiation codon ATG of yeast *PGK* gene [S. M. Kingsman, *Nucleic Acids Res.* **10**, 2625 (1982); R. A. Hitzeman *et al.*, *ibid.*, p. 7791] was converted to a blunt end with T4 polymerase, then ligated with the Nsi I linker (TGCATGCATGCA) in order to generate the Sph I site that overlaps with the initiation codon. The resulting plasmid was digested by Sph I and treated with T4 polymerase to generate the blunt end just after the ATG. A Bgl II linker (GCAGATCTGC) or the artificial intron fragment (previously ligated with the Bgl II linker at the 3′ end) was ligated to the blunt end. The fragments (1.7 kb and 1.6 kb) containing the *PGK* promoter and ATG with or without the intron were released by Hind III and Bgl II, then ligated into the Hind III–Bam HI site of plasmid pFN8 (*25*), which contains the *Escherichia coli lacZ* gene (from the eighth codon to the end) and *URA3* as a selectable marker. The resulting plasmids were used to transform the yeast strain NY6A (*MATa*, *ura3-52*, *leu2-3*, *leu2-112*, *his4-519*).
16. A 33-bp Pst I–Sna BI fragment containing the 5′ consensus sequence 5′ GTATGT 3′ was released from the pUC-AI plasmid (Fig. 1). This fragment was inserted between Pst I and Klenow-treated Xba I sites of the pUC-AI plasmid. The resulting plasmid is called pUC AI-1.
17. A 38-bp Sac I–Pvu II fragment containing the TACTAAC box was released from the pUC-AI plasmid (Fig. 1). This fragment was inserted between Sac I and Klenow-treated Xba I sites of the pUC-AI plasmid. The resulting plasmid is called pUC AI-2.
18. Plasmid pUC AI-1 (*16*) was digested with Pst I and then treated with "Slow" Bal 31 nuclease (Takara shuzo) for 5 to 120 s. Xba I linkers were added to the ends, and the fragment containing the 3′ half of the intron was released by Xba I and Sca I, then ligated with the Xba I–Sca I fragment of pUC-AI (which contains the 5′ half of the intron).
19. Plasmid pUC AI-2 (*17*) was digested with Sal I and then treated with "Slow" Bal 31 nuclease as described (*18*). Xba I linkers were added, and the fragment containing the 5′ half of the intron was released by Xba I and Sca I, then ligated with the Xba I–Sca I fragment of pUC-AI, which contains the 3′ half of the intron.
20. Each plasmid containing a deleted intron was used to transform the yeast strain NY6A. Transformants were transferred to X-gal plates (*24*). Plates were then incubated either at 36°C or at 16°C for 3 days.
21. *AI-3* retains a 13-bp inverted repeat. *AI-1* derivatives retaining 17-bp and longer or 10-bp and shorter inverted repeats were not cold-sensitive (*14*). *AI-4* retains a 17-bp inverted repeat. *AI-2* derivatives retaining 21-bp and longer or 13-bp and shorter inverted repeats were not cold sensitive (*14*).
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26. We thank K. Miyoshi and T. Kawazoe for providing oligonucleotides, and Y. Shimura, D. A. Shub, and S. Ohno for critical reading of the manuscript.

22 December 1988; accepted 28 March 1989

Isolation of Single-Copy Human Genes from a Library of Yeast Artificial Chromosome Clones

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A recently developed cloning system based on the propagation of large DNA molecules as linear, artificial chromosomes in the yeast *Saccharomyces cerevisiae* provides a potential method of cloning the entire human genome in segments of several hundred kilobase pairs. Most applications of this system will require the ability to recover specific sequences from libraries of yeast artificial chromosome clones and to propagate these sequences in yeast without alterations. Two single-copy genes have now been cloned from a library of yeast artificial chromosome clones that was prepared from total human DNA. Multiple, independent isolates were obtained of the genes encoding factor IX and plasminogen activator inhibitor type 2. The clones, which ranged in size from 60 to 650 kilobases, were stable on prolonged propagation in yeast and appear to contain faithful replicas of human DNA.

IN APPLICATIONS THAT REQUIRE THE analysis of large tracts of genomic DNA, the yeast artificial chromosome (YAC) cloning system has a number of potential advantages over conventional cloning methods (*1*). Its open-ended capacity for large inserts has allowed the cloning of segments of human DNA that are ten times larger than those that can be cloned in cosmids (*1, 2*). As a eukaryotic host, yeast provides a substantially different environment than *Escherichia coli* in which to propagate the DNA of higher organisms. The ease

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